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molecules present to cause nonspecific binding of the protein A component). Subsequent addition of a labeled avidin molecule binds to the biotinylated protein A, completing the formation of a detection complex (Jagannath and Sehgal, 1989).

To develop assay systems using the avidin–biotin interaction, it is first necessary to produce the associated avidin conjugates and/or biotinylated components. When the LAB technique is employed, the avidin conjugate is made using cross-linking agents, not biotinylation reagents, in order to maintain the binding capacity of the avidin tetramer toward other biotinylated molecules. In the BRAB assay system, avidin is left unconjugated and acts merely as the multivalent bridging molecule, while both the targeting molecule and the detection molecule are biotinylated. The components for the ABC assay are identical to those of the BRAB system.

The following sections discuss the main techniques used to make avidin (or streptavidin) conjugates and various biotinylated components. Chapter 8, Section 3, should be consulted for a complete overview of the biotinylation reagents currently available.

3. Preparation of Avidin or Streptavidin Conjugates

Conjugates of avidin or streptavidin with other protein molecules must be prepared to design systems using the LAB assay technique. Suitable protein molecules attached to avidin or streptavidin either possess indigenous detectability, such as in the case of ferritin or phycobiliproteins, or possess catalytic activity (enzymatic) that can be utilized to produce a detectable substrate product. The majority of conjugation procedures for making avidin–protein or streptavidin–protein conjugates use the amines, sulfhydryls, and carbohydrates on each protein as functional groups for cross-linking.

Perhaps the most common conjugates of avidin or streptavidin involve attaching enzyme molecules for use in ELISA systems. As in the case of antibody–enzyme conjugation schemes (Chapter 10), by far the most commonly used enzymes for this purpose are horseradish peroxidase and alkaline phosphatase. Other enzymes such as β-galactosidase and glucose oxidase are used less often, especially with regard to assay tests for clinically important analytes (Chapter 16).

Other proteins commonly cross-linked to avidin or streptavidin are chromogenic or fluorescent molecules, such as ferritin or phycobiliproteins (Chapter 8, Section 1.7). These conjugates can be used in microscopy techniques to stain and localize certain antigens or receptors in cells or tissue sections.

The following sections discuss three main methods for preparing these types of avidin- or streptavidin–protein conjugates. They involve using an NHS ester–maleimide heterobifunctional cross-linker, making use of the carbohydrate on glycoproteins for reductive amination coupling, and employing the old technique of homobifunctional cross-linking with glutaraldehyde.

3.1. NHS Ester–Maleimide-Mediated Conjugation Protocols

Heterobifunctional cross-linking agents can be used to control the degree of protein conjugation, thus limiting polymerization and controlling the molar ratio of each component in the final complex (Chapter 5). Particularly useful heterobifunctionals

include the amine- and sulfhydryl-reactive NHS ester–maleimide cross-linkers discussed in Chapter 5, Section 1. Chief among these is SMCC or sulfo-SMCC (Chapter 5, Section 1.3), which contains a reasonably long spacer and an extremely stable maleimide group due to the adjacent cyclohexane ring in its cross-bridge.

Conjugations done with SMCC usually involve up to 3 steps. In the first stage, one of the proteins is modified at its amine groups via the NHS ester end of the cross-linker to form amide linkages terminating in active maleimide groups. If the other protein to be conjugated does not contain sulfhydryl residues necessary to react with the maleimide-activated protein, it must be modified to contain them (Chapter 1, Section 4.1). Finally, the two reactive components are mixed together in the proper ratio to effect the conjugation reaction.

For the preparation of avidin–enzyme conjugates, either protein may be first modified with SMCC and the other one modified to contain —SH groups. Since avidin or streptavidin does not possess any free sulfhydryls—and the disulfides present in avidin are inaccessible to reduction—these proteins must be modified with either the cross-linker or with a thiolating agent before conjugation. If the enzyme employed contains free sulfhydryls in its native state, such as β-galactosidase, then it is convenient to activate avidin with SMCC and simply add the sulfhydryl-containing protein to it for conjugation. If the enzyme does not contain free sulfhydryls (as is the case with alkaline phosphatase or horseradish peroxidase), then the choice of which component gets maleimide-activated and which gets thiolated is up to the individual.

The following protocol describes the activation of avidin or streptavidin with sulfo-SMCC and its subsequent conjugation with an enzyme modified to contain sulfhydryls using SATA (Chapter 1, Section 4.1). A method for the opposite approach, wherein the enzyme is activated with SMCC and the avidin component is thiolated, is presented immediately after this protocol. This strategy may be the most common approach to forming these conjugates (Fig. 363). In addition, since there are enzymes commercially available that are preactivated with SMCC (Pierce), their use may be the easiest solution.

Protocol for the Conjugation of SMCC-Activated Avidin or Streptavidin with Thiolated Enzyme

Activation of Avidin or Streptavidin with SMCC

1. Dissolve avidin or streptavidin (Pierce) in 0.1 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.2, at a concentration of 10 mg/ml.
2. Add 1 mg of sulfo-SMCC (Pierce) to each milliliter of avidin or streptavidin solution. Mix to dissolve.
3. React for 30–60 min at room temperature. Since maleimide groups are labile in aqueous solution, extended reaction times should be avoided.
4. Immediately purify the maleimide-activated avidin or streptavidin away from excess cross-linker and reaction by-products by gel filtration on a desalting column (Sephadex G-25 or the equivalent). Use 0.1 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.2, as the chromatography buffer. Pool the fractions containing protein (the first peak eluting from the column). After elution, adjust the protein concentration to 10 mg/ml for the conjugation reaction (centrifugal concentrators work well for this step). At this point, the maleimide-activated avidin may be frozen and lyophilized to preserve its maleimide activity. The modified pro-

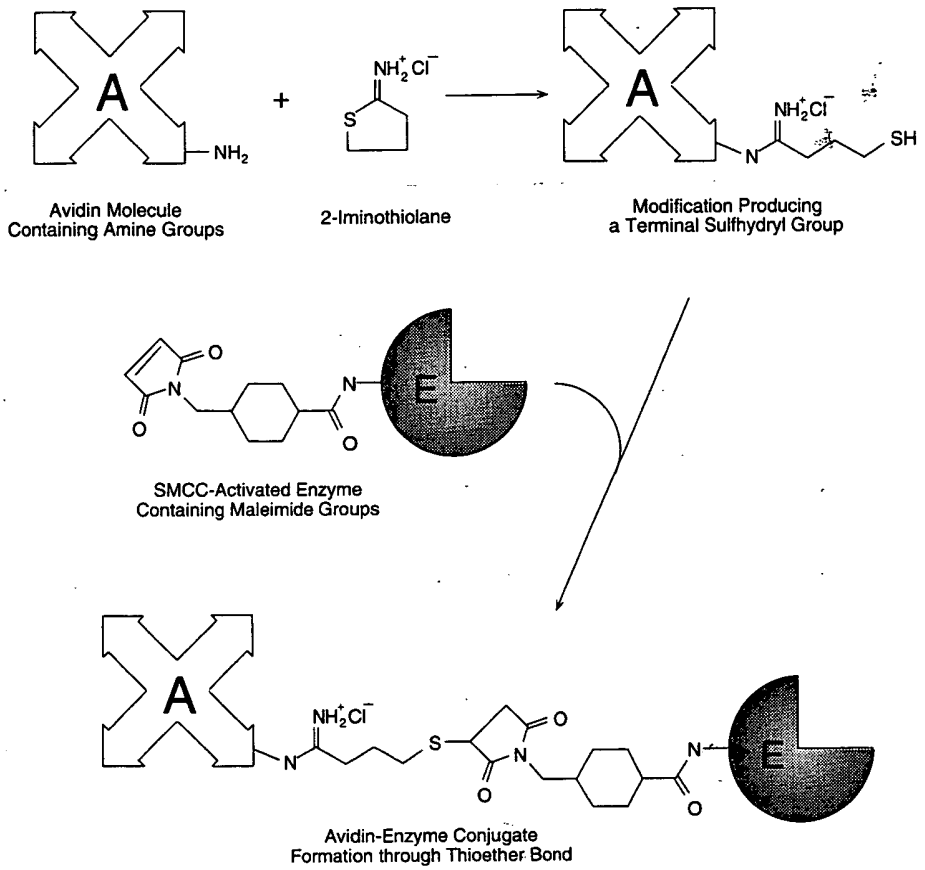

Figure 363 Avidin may be modified with 2-iminothiolane to produce sulfhydryl groups. Subsequent reaction with a maleimide-activated enzyme produces a thioether-linked conjugate.

tein is stable for at least 1 year in a freeze-dried state. If kept in solution, the maleimide-activated avidin is labile and should be used immediately to conjugate with a thiolated enzyme following the procedure described below.

Modification of Enzyme with SATA

If β-galactosidase is used to conjugate with an SMCC-activated avidin or streptavidin, then there is no need to thiolate the enzyme, since it contains sulfhydryls in its native state (Sivakoff and Janes, 1988; Fujiwara *et al.*, 1988). For conjugations using horseradish peroxidase, alkaline phosphatase, or glucose oxidase, however, thiolation is necessary to add the requisite sulfhydryls.

1. Dissolve the enzyme to be modified in 0.1 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.2, at a concentration of 10 mg/ml.
2. Prepare a stock solution of SATA (Pierce) by dissolving it in DMSO at a concentration of 13 mg/ml. Use a fume hood to handle the organic solvent.
3. Add 25 µl of the SATA stock solution to each milliliter of 10 mg/ml enzyme

solution. For different concentrations of enzyme in the reaction medium, proportionally adjust the amount of SATA addition; however, do not exceed 10% DMSO in the aqueous reaction medium.

4. React for 30 min at room temperature.
5. To purify the SATA-modified enzyme perform a gel filtration separation using Sephadex G-25 or dialyze against 0.1 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.2, containing 10 m*M* EDTA. Purification is not absolutely required, since the following deprotection step is done using hydroxylamine at a significant molar excess over the initial amount of SATA added. Whether a purification step is done or not, at this point, the derivative is stable and may be stored under conditions that favor long-term enzyme activity.
6. Deprotect the acetylated sulfhydryl groups on the SATA-modified enzyme according to the following protocol:
   a. Prepare a 0.5 *M* hydroxylamine solution in 0.1 *M* sodium phosphate, pH 7.2, containing 10 m*M* EDTA.
   b. Add 100 μl of the hydroxylamine stock solution to each milliliter of the SATA-modified enzyme. Final concentration of hydroxylamine in the enzyme solution is 50 m*M*.
   c. React for 2 h at room temperature.
   d. Purify the thiolated enzyme by gel filtration on Sephadex G-25 using 0.1 *M* sodium phosphate, 0.1 *M* NaCl, pH 7.2, containing 10 m*M* EDTA as the chromatography buffer. To obtain efficient separation between the thiolated protein and excess hydroxylamine and reaction by-products, the sample size applied to the column should be at a ratio of no more than 5% sample volume to the total column volume. Collect 0.5-ml fractions. Pool the fractions containing protein by measuring the absorbance of each fraction at 280 nm.

Production of Conjugate

1. Immediately mix the thiolated enzyme with an amount of maleimide-activated avidin (or streptavidin) to obtain the desired molar ratio of enzyme to avidin in the conjugate. Use of a 4:1 (enzyme:avidin) molar ratio in the conjugation reaction usually results in high-activity conjugates suitable for use in many enzyme-linked immunoassay procedures employing the LAB approach.
2. React for 30–60 min at 37°C or 2 h at room temperature. The conjugation reaction also may be done at 4°C overnight.

A variation of the above method can be used, wherein the enzyme is first activated with SMCC and conjugated to a thiolated avidin or streptavidin molecule. This approach probably is the most common way of preparing avidin–enzyme conjugates, and since the preactivated enzymes are readily available (Pierce), it also may be the easiest.

Protocol for the Conjugation of SMCC-Activated Enzymes with Thiolated Avidin or Streptavidin

Activation of Enzyme with Sulfo-SMCC

The following protocol describes the activation of HRP with sulfo-SMCC. Other enzymes may be activated in a similar manner. The activated enzyme possesses male-

imide groups that are relatively unstable in aqueous solution. Therefore, the thiolation reaction (part B) should be coordinated with the activation process so that the conjugation (part C) can be done immediately. Note that if preactivated enzymes are obtained, this step may be eliminated.

1. Dissolve HRP in 0.1 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.2, at a concentration of 10 mg/ml.
2. Add 3.3 mg of sulfo-SMCC (Pierce) to each milliliter of the HRP solution. Mix to dissolve and react for 30 min at room temperature. Alternatively, two equal additions of cross-linker may be done—the second one after 15 min of incubation—to obtain even more efficient modification.
3. Immediately purify the maleimide-activated HRP away from excess cross-linker and reaction by-products by gel filtration on a desalting column (Sephadex G-25 or the equivalent). Use 0.1 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.2, as the chromatography buffer. HRP can be observed visually as it flows through the column due to the color of its heme ring. Pool the fractions containing the HRP peak. After elution, adjust the HRP concentration to 10 mg/ml for the conjugation reaction. At this point, the maleimide-activated enzyme may be frozen and lyophilized to preserve its maleimide activity. The modified enzyme is stable for at least 1 year in a freeze-dried state. If kept in solution, the maleimide-activated HRP should be used immediately to conjugate with thiolated avidin or streptavidin following the protocols outlined below.

Thiolation of Avidin or Streptavidin

1. Dissolve avidin or streptavidin in 0.1 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.2, at a concentration of 10 mg/ml.
2. Prepare a stock solution of SATA by dissolving it in DMSO at a concentration of 13 mg/ml. Use a fume hood to handle the organic solvent.
3. Add 25 µl of the SATA stock solution to each milliliter of 10 mg/ml avidin or streptavidin solution. For different concentrations of protein in the reaction medium, proportionally adjust the amount of SATA addition; however, do not exceed 10% DMSO in the aqueous reaction medium.
4. React for 30 min at room temperature.
5. To purify the SATA-modified avidin or streptavidin use gel filtration on a column of Sephadex G-25 or dialyze against 0.1 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.2, containing 10 m*M* EDTA. At this point, the derivative is stable and may be stored under conditions that favor long-term avidin activity.
6. Deprotect the acetylated sulfhydryl groups on the SATA-modified protein according to the following protocol:
   a. Prepare a 0.5 *M* hydroxylamine solution in 0.1 *M* sodium phosphate, pH 7.2, containing 10 m*M* EDTA.
   b. Add 100 µl of the hydroxylamine stock solution to each milliliter of the SATA-modified avidin or streptavidin. Final concentration of hydroxylamine in the solution is 50 m*M*.
   c. React for 2 h at room temperature.
   d. Purify the thiolated protein by gel filtration on Sephadex G-25 using 0.1 *M* sodium phosphate, 0.1 *M* NaCl, pH 7.2, containing 10 m*M* EDTA as the chromatography buffer.

Conjugation of SMCC-Activated Enzyme with Thiolated Avidin or Streptavidin

1. Immediately mix the SMCC-activated enzyme with an amount of thiolated avidin (or streptavidin) to obtain the desired molar ratio of enzyme to avidin in the conjugate. Use of a 4:1 (enzyme:avidin) molar ratio in the conjugation reaction usually results in high-activity conjugates suitable for use in many enzyme-linked immunoassay procedures employing the LAB approach.
2. React for 30–60 min at 37°C or 2 h at room temperature. The conjugation reaction also may be done at 4°C overnight.

3.2. Periodate Oxidation/Reductive Amination Conjugation Protocols

Glycoproteins may be conjugated with another amine-containing protein through the process of periodate oxidation and reductive amination. Periodate oxidation of polysaccharide components on the glycoprotein results in the formation of reactive aldehyde residues by cleavage of carbon—carbon bonds and oxidation of the associated adjacent hydroxyls (Chapter 1, Section 4.4). Conjugation with another protein may be done by reacting the aldehydes with amines to form intermediate Schiff bases with subsequent reduction using sodium cyanoborohydride to create stable secondary amine bonds.

This method of conjugation is particularly well suited to coupling HRP or ferritin with avidin or streptavidin. Both HRP and avidin are glycoproteins that can be oxidized with sodium periodate to generate aldehydes. Thus, HRP–avidin, HRP–streptavidin, and ferritin–avidin may be prepared by reductive amination. Ferritin is a large, complex protein of molecular weight 750,000. Its structure is made of a protein shell of diameter approximately 12 nm that surrounds a micelle core consisting of ferric hydroxide of about 6 nm in diameter. This core contains more than 2000 iron atoms, making the protein extremely electron dense and thus perfect for electron microscopy applications. The properties of HRP are described in Chapter 16, Section 1.

The following protocol is adapted from Bayer *et al.* (1976).

Protocol for the Conjugation of Avidin with Ferritin Using Reductive Amination

1. Dissolve avidin (Pierce) in 0.1 *M* sodium acetate, 0.15 *M* NaCl, pH 4.5, at a concentration of 3 mg/ml.
2. Dissolve ferritin (Sigma) in 0.1 *M* sodium acetate, 0.15 *M* NaCl, pH 4.5, at a concentration of 100 mg/ml.
3. Add 1 ml of ferritin solution to every 5 ml of avidin solution. Chill on ice.
4. Dissolve sodium periodate in water at a concentration of 100 m*M*. Prepare fresh and protect from light.
5. Add 110 μl of sodium periodate solution to each milliliter of avidin/ferritin solution.
6. React for 3 h on ice with periodic mixing. Protect from light.
7. Remove excess periodate by gel filtration on a column of Sephadex G-25 or by overnight dialysis against 50 m*M* sodium borate, 0.15 *M* NaCl, pH 8.5.

8. Dissolve 10 mg of sodium borohydride in 1 ml of 10 m*M* NaOH. Prepare fresh. Add 83 µl of this reducing solution to each ml of avidin/ferritin solution.
9. React for 1 h on ice.
10. Remove excess reductant by gel filtration using a column of Sephadex G-25 or by extensive dialysis against 20 m*M* sodium phosphate, 0.15 *M* NaCl, pH 7.4.

Conjugation of HRP by reductive amination can be done by oxidizing the carbohydrate on the enzyme and subsequently coupling to the amines on avidin or streptavidin (Fig. 364).

Protocol for the Preparation of Avidin–HRP or Streptavidin–HRP by Reductive Amination

Oxidation of HRP with Sodium Periodate

1. Dissolve HRP in water or 0.01 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.2, at a concentration of 10–20 mg/ml.
2. Dissolve sodium periodate in water at a concentration of 0.088 *M*. Protect from light.
3. Immediately add 100 µl of the sodium periodate solution to each ml of the HRP solution. This results in an 8 m*M* periodate concentration in the reaction mixture. Mix to dissolve. Protect from light.
4. React in the dark for 20 min at room temperature. A color change will be

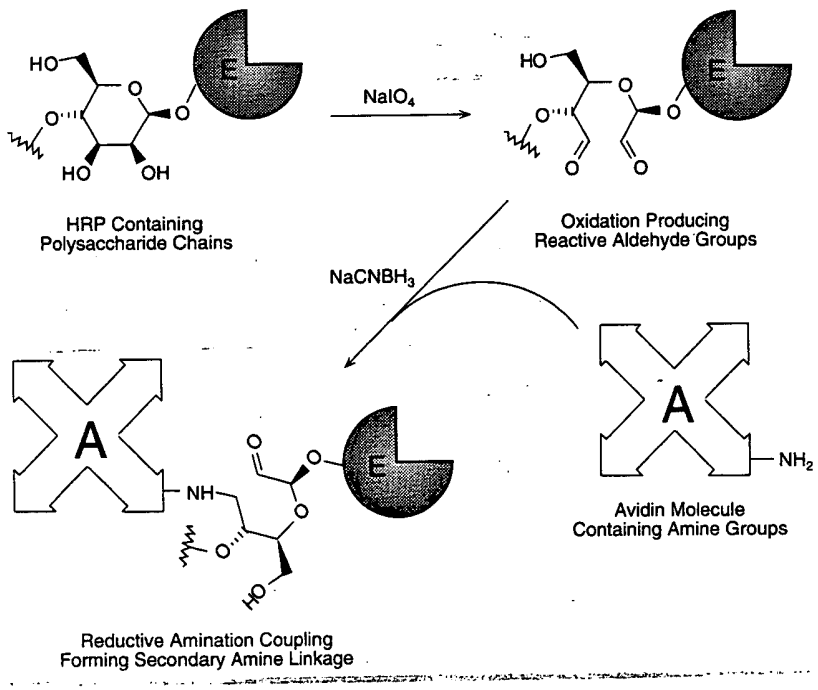

Figure 364 Oxidation of the polysaccharide components of HRP produces reactive aldehyde groups. Conjugation to avidin then may be done by reductive amination.

apparent as the reaction proceeds—changing from the brownish/gold color of concentrated HRP to green.

5. Immediately purify the oxidized enzyme by gel filtration using a column of Sephadex G-25. The chromatography buffer is 0.01 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.2. Collect 0.5-ml fractions and monitor for protein at 280 nm. HRP also may be detected by its absorbance at 403 nm. In oxidizing large quantities of HRP, the fraction collection process may be done visually—just pooling the colored HRP peak as it comes off the column.
6. Pool the fractions containing protein. Adjust the enzyme concentration to 10 mg/ml for the conjugation step. The periodate-activated HRP may be stored frozen or freeze-dried for extended periods without loss of activity. However, do not store the preparation in solution at room temperature or 4°C, since precipitation will occur over time due to self-polymerization.

Conjugation of Periodate-Oxidized HRP with Avidin or Streptavidin

1. Dissolve avidin or streptavidin at a concentration of 10 mg/ml in 0.2 *M* sodium bicarbonate, pH 9.6, at room temperature. The high-pH buffer will result in very efficient Schiff base formation and conjugation with the highest possible incorporation of enzyme molecules per avidin or streptavidin molecule. To produce lower molecular weight conjugates (using less efficient Schiff base formation conditions), dissolve the proteins at a concentration of 10 mg/ml in 0.1 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.2.
2. The periodate-oxidized HRP (prepared above) was finally purified using 0.01 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.2. For conjugation using the lower pH buffered environment, this HRP preparation can be used directly at 10 mg/ml concentration. For conjugation using the higher pH carbonate buffer, dialyze the HRP solution against 0.2 *M* sodium carbonate, pH 9.6, for 2 h at room temperature prior to use.
3. Mix the avidin or streptavidin solution with the enzyme solution at a ratio of 1:6.6 (v/v). Since avidin has a molecular weight of about 66,000 and the molecular weight of HRP is 40,000, this ratio of volumes will result in a molar ratio of HRP:avidin equal to 4:1. For conjugates consisting of greater enzyme-to-avidin ratios, proportionally increase the amount of enzyme solution as required. Typically, molar ratios of 2:1 to 10:1 (enzyme:avidin) give acceptable conjugates useful in a variety of ELISA techniques.
4. React for 2 h at room temperature to form the initial Schiff base interactions.
5. In a fume hood, add 10 µl of 5 *M* sodium cyanoborohydride (Sigma) per milliliter of reaction solution. Caution: cyanoborohydride is extremely toxic. All operations should be done with care in a fume hood. Also, avoid any contact with the reagent, as the 5 *M* solution is prepared in 1 *N* NaOH.
6. React for 30 min at room temperature (in a fume hood).
7. Block unreacted aldehyde sites by addition of 50 µl of 1 *M* ethanolamine, pH 9.6, per milliliter of conjugation solution. Approximately a 1 *M* ethanolamine solution may be prepared by addition of 300 µl ethanolamine to 5 ml of deionized water. Adjust the pH of the ethanolamine solution by addition of concentrated HCl, keeping the solution cool on ice.

8. React for 30 min at room temperature.
9. Purify the conjugate from excess reactants by dialysis or gel filtration using Sephadex G-25. Use 0.01 *M* sodium phosphate, 0.15 *M* NaCl, pH 7, as the buffer for either operation. Use a fume hood, since cyanoborohydride will be present in some of the fractions.

3.3. Glutaraldehyde Conjugation Protocol

Glutaraldehyde is one of the oldest homobifunctional reagents used for protein conjugation. It reacts with amine groups to create cross-links by one of several routes (Chapter 4, Section 6.2). Under reducing conditions, the aldehydes on both ends of glutaraldehyde will couple with amines to form secondary amine linkages. The reagent is highly efficient at protein conjugation, but has a tendency to form high-molecular-weight polymers due to its homobifunctional nature. Single-step protocols using glutaraldehyde are particularly notorious at resulting in some degree of insoluble protein oligomers (Porstmann *et al.*, 1985). Two-step methods somewhat alleviate this problem, but the potential for conjugate precipitation is still present.

Preparation of avidin or streptavidin conjugates with other proteins can be accomplished using either a one- or a two-step glutaraldehyde procedure. Both methods may result in some degree of oligomer formation; however, the two-step protocol may keep insolubles to a minimum. Although the following procedures are described using particular proteins, they may be used as a general guide for coupling enzymes, ferritin, phycobiliproteins, or other detectable proteins to avidin or streptavidin. Some optimization may be necessary to obtain the best yield of active conjugate.

Protocol for the One-Step Glutaraldehyde Conjugation of Ferritin to Avidin or Streptavidin

This protocol is adapted from Bayer and Wilchek (1980).

1. Prepare a solution containing 5 mg/ml avidin (or streptavidin) and 25 mg/ml ferritin in 0.02 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.4, at room temperature. Note: for the coupling of other proteins to avidin or streptavidin, their concentration may be reduced from the 25 mg/ml stated for ferritin.
2. In a fume hood, add 10 µl of 25% glutaraldehyde per milliliter of avidin/ferritin solution. Mix well.
3. React for 1 h at room temperature.
4. To reduce the resultant Schiff bases and any excess aldehydes, add sodium borohydride to a final concentration of 10 mg/ml.

Note: Some protocols do not call for a reduction step. As an alternative to reduction, add 50 µl of 0.2 *M* lysine in 0.5 *M* sodium carbonate, pH 9.5, to each milliliter of the conjugation reaction to block excess reactive sites. Block for 2 h at room temperature. Other amine-containing small molecules may be substituted for lysine—such as glycine, Tris buffer, or ethanolamine.

5. Reduce for 1 h at 4°C.
6. To remove any insoluble polymers that may have formed, centrifuge the conjugate or filter it through a 0.45-µm filter. Purify the conjugate by gel filtration or dialysis using PBS, pH 7.4.

A two-step glutaraldehyde protocol may result in lower molecular weight conjugates, thus limiting the degree of insoluble material formed during the cross-linking process. The following protocol is adapted from Avrameas (1969).

Protocol for the Two-Step Glutaraldehyde Conjugation of Enzymes to Avidin or Streptavidin

1. Dissolve the enzyme at a concentration of 10 mg/ml in 0.1 *M* sodium phosphate, 0.15 *M* NaCl, pH 6.8.
2. Add glutaraldehyde to a final concentration of 1.25%.
3. React overnight at room temperature.
4. Purify the activated enzyme from excess glutaraldehyde by gel filtration (using Sephadex G-25) or by dialysis against PBS, pH 6.8.
5. Dissolve avidin or streptavidin at a concentration of 10 mg/ml in 0.5 *M* sodium carbonate, pH 9.5. Mix the activated enzyme with the avidin or streptavidin solution at the desired molar ratio to effect the conjugation. Mixing the equivalent of 1–2 mol of enzyme per mole of avidin usually results in acceptable conjugates.
6. React overnight at 4°C.
7. To reduce the resultant Schiff bases and any excess aldehydes, add sodium borohydride to a final concentration of 10 mg/ml.

Note: Some protocols avoid a reduction step. As an alternative to reduction, add 50 µl of 0.2 *M* lysine in 0.5 *M* sodium carbonate, pH 9.5, to each milliliter of the conjugation reaction to block excess reactive sites. Block for 2 h at room temperature. Other amine-containing small molecules may be substituted for lysine—such as glycine, Tris buffer, or ethanolamine.

8. Reduce for 1 h at 4°C.
9. To remove any insoluble polymers that may have formed, centrifuge the conjugate or filter it through a 0.45-µm filter. Purify the conjugate by gel filtration or dialysis using PBS, pH 7.4.

4. Preparation of Fluorescently Labeled Avidin or Streptavidin

Fluorophore modification of avidin or streptavidin creates a reagent system that can be used to detect and localize biotinylated targeting molecules. The application of such reagents in immunohistochemical staining techniques is significant (Bonnard *et al.*, 1984). A biotinylated antibody directed against a particular tissue antigen can be allowed to bind its target *in situ*, and then a fluorescently tagged avidin or streptavidin may be added to bind and visualize the antibody-bound antigenic sites by luminescence. Individual cellular structures can be labeled in similar assay strategies and detected by fluorescent microscopy or cell sorting techniques (Sternberger, 1986; Abou-Samra *et al.*, 1990). Biotinylated targeting molecules like antibodies usually possess low nonspecific binding potential despite the presence of a biotin tag. Thus, background fluorescence of tissue sections can be kept to a minimum using avidin–biotin detection. The multivalent nature of avidin or streptavidin combined with the potential of more than one biotin tag per antibody creates a system of much greater potential sensitivity than when using fluorescently modified antibodies directly. The